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Molecules and Cells

Antioxidative Role of Selenoprotein W in Oxidant-Induced Mouse Embryonic Neuronal Cell Death

Youn Wook Chung[1,2,5], Daewon Jeong[3], Ok Jeong Noh[1], Yong Hwan Park[1], Soo Im Kang[1,6], Min Goo Lee[1], Tae-Hoon Lee[4], Moon Bin Yim[2], and Ick Young Kim[1,*]

It has been reported that selenoprotein W (SelW) mRNA is highly expressed in the developing central nerve system of rats, and its expression is maintained until the early postnatal stage. We here found that SelW protein significantly increased in mouse brains of postnatal day 8 and 20 relative to embryonic day 15. This was accompanied by increased expression of SOD1 and SOD2. When the expression of SelW in primary cultured cells derived from embryonic cerebral cortex was knocked down with small interfering RNAs (siRNAs), SelW siRNA-transfected neuronal cells were more sensitive to the oxidative stress induced by treatment of H_2O_2 than control cells. TUNEL assays revealed that H_2O_2-induced apoptotic cell death occurred at a higher frequency in the siRNA-transfected cells than in the control cells. Taken together, our findings suggest that SelW plays an important role in protection of neurons from oxidative stress during neuronal development.

INTRODUCTION

Selenium is inserted into selenoenzymes as selenocysteine (Sec) (Cone et al.,1976). Sec is the 21st amino acid and is encoded by the UGA 'STOP' codon (Zinoni et al., 1986). In eukaryotes, a specific RNA stem loop structure, the selenocysteine-insertion-sequence (SECIS), is required for Sec insertion (Berry et al., 1991). In addition, the SECIS-binding protein SBP2 (Hubert et al., 1996), the Sec-specific translation factor EF-Sec (Fagegaltier et al., 2000; Tujebajeva et al., 2000), and the selenocysteine-specific $tRNA^{Sec}$ (Lee et al., 1989) are needed for selenoprotein translation. Twenty-five selenoprotein-encoding genes have been identified in mammals (Kryukov et al., 2003), and several functions of selenoproteins have been described. Glutathione peroxidase (GPx) (Flohe et al., 1973; Rotruck et al., 1973) and thioredoxin reductase (TR) (Mustacich and Powis, 2000) protect cells from oxidative stress, and they may have a role in brain function (Brigelius-Flohe, 1999; Hill et al., 1997; Lovell et al., 2000; Trepanier et al., 1996). Selenoprotein P (SelP), the major selenoprotein in blood plasma, serves as phospholipid hydroperoxide reductase and as a selenium supply protein (Burk and Hill, 2005). Methionine-*R*-sulfoxide reductase 1 (MsrB1) (previously reported as selenoprotein R or selenoprotein X) reduces methionine sulfoxide to methionine (Kim and Gladyshev, 2007). Selenoprotein T (SelT), which is mainly localized in the endoplasmic reticulum, is involved in the regulation of calcium homeostasis and neuroendocrine secretion (Grumolato et al., 2008). Selenoprotein H (SelH) is a novel nucleolar oxidoreductase (Novoselov et al., 2007). Several selenoproteins with unknown functions also have been detected in the brain. For example, selenoprotein N (SelN), the first selenium-containing protein reported to be associated to a genetic disorder, is expressed in the brain and is located in the endoplasmic reticulum as a glycoprotein (Ferreiro et al., 2002; Moghadaszadeh et al., 2001; Petit et al., 2003). Selenoprotein M (SelM), 15-kDa selenoprotein (Sel15), and selenoprotein W (SelW) mRNA were also detected in the brain (Gu et al., 2000; Korotkov et al., 2002; Kumaraswamy et al., 2002).

SelW is localized primarily in cytosolic compartments, although a small quantity is found the cytoplasmic membrane (Vendeland et al., 1993; Yeh et al., 1995). To date, the biological activity of SelW has not been identified. However, we and others have reported that the cysteine^{37} (Cys^{37}) of SelW can be glutathiolated (Beilstein et al., 1996; Gu et al., 1999). In addition, cells that overexpress SelW were found to be more resistant to oxidative stress, suggesting a potential role as an antioxidant (Jeong et al., 2002). Recently, it has been suggested that SelW is a member of the thioredoxin family, since it is involved in redox regulation through its interactions with 14-3-3 proteins (Aachmann et al., 2007; Dikiy et al., 2007). Levels of selenoproteins are strongly dependent on selenium concentrations, and selenium-deficient diets lead to a decrease in the expression of most selenoproteins in various tissues (Allan et al., 1999; Gu et

[1]Laboratory of Cellular and Molecular Biochemistry, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea, [2]Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 50, 9000 Rockville Pike, Bethesda, MD 20892, USA, [3]Department of Microbiology and Aging-associated Disease Research Center, Yeungnam University College of Medicine, Daegu 705-030, Korea, [4]School of Dentistry, Dental Science Research Institute, The 2nd Stage of Brain Korea 21 for the Dental School, Chonnam National University, Gwangju 500-757, Korea, [5]Present address: Translational Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, 9000 Rockville Pike, Bethesda, MD 20892, USA, [6]Present address: Genetic Disease Research Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 10, 9000 Rockville Pike, Bethesda, MD 20892, USA
*Correspondence: ickkim@korea.ac.kr

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al., 1999; Yeh et al., 1997a; 1997b). However, SelW expression in the brain was maintained at a reasonable level in the selenium-deficient conditions (Yeh et al., 1997b). More specifically, *selW* mRNA was expressed at high levels in the cortex, dentate gyrus, and hippocampus of postnatal rat brains (Jeong et al., 2004). These observations suggest that selenium and SelW play an important role in the brain function and during neuronal development. However, the mechanisms by which SelW expression is regulated during embryonic and postnatal stages, and the mechanisms by which it exerts its antioxidant activity in the neuronal cells are not fully understood.

In the present study, we compared the expression patterns of SelW with other selenoproteins and non-selenium-containing antioxidant proteins in the mouse brain at different stages of development. And, to verify its function, we knocked down SelW expression using a small interfering RNA (siRNA) technique.

MATERIALS AND METHODS

Reagents

Poly-D-lysine, laminin, formaldehyde, and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma (USA).

Primary cell culture

Primary cultures of mouse embryo-driven neuronal cells were prepared as described previously (Moon et al., 2008; Niethammer et al., 2000). In brief, embryonic neuronal cells were isolated from the cerebral cortex of ICR mice at the embryonic stage day 15 (E15) (Orient, Korea), seeded onto poly-D-lysine/laminin-coated 6-well plates, and grown in Neurobasal medium (Invitrogen, USA) containing B27 and GlutaMAX-1 supplement (Invitrogen) and antibiotics.

Real-time PCR analysis

Total RNA was extracted from brains of E15 and P8 mice using TRIzol (Invitrogen). First-strand cDNAs were synthesized by using ThermoScript RT-PCR system (Invitrogen), following the manufacturer's manual. Quantitative real-time PCR was performed using the 7900H sequence detection system (Applied Biosystems, USA). Primers and TaqMan probes used to detect the indicated murine genes were purchased from Applied Biosystems and included the following: SelW, Mm00486050_m1; Thioredoxin (Trx), Mm00726847_s1; TR1, Mm00443675_m1; GPx1, Mm00656767_g1; Catalase (CAT), Mm00437992_m1; Superoxide anion dismutase 1 (SOD1), Mm01700393_g1; SOD2, Mm00449726_m1; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Mm99999915_g1.

Immunoblot analysis

The brains removed the ICR mice were washed with PBS and homogenized in a solution containing 10 mM Hepes-NaOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, aprotinin (2 μg/ml), leupeptin (2 μg/ml), and 0.3% Nonidet P-40. After centrifugation, supernatants were fractionated via SDS-polyacrylamide gel electrophoresis and then subjected to immunoblot analysis with mouse monoclonal antibodies to SelW (Jeong et al., 2002), Thioredoxin (Trx; kindly provided from SG Rhee, NIH, USA), Thioredoxin Reductase 1 (TR1; Abcam), Glutathione Peroxidase (GPx1; Abcam), Catalase (Cat; Fitzgerald), Superoxide Dismutase 1 (SOD1; Fitzgerald), Superoxide Dismutase 2 (SOD2; Fitzgerald), and β-actin (Sigma). The blots were visualized using enhanced chemiluminescence (Supersignal, Pierce).

siRNA preparation and transfection

The siRNA corresponding to the *selW* gene was designed and synthesized by Invitrogen (Stealth RNAi). The sequence for the selW siRNA was 5′-GGA TAC AGA GAG CAC GTT CCG GAA A-3′. Three days after plating, the primary neurons were transfected with siRNA using LipofectAMINE 2000 (Invitrogen) in serum-free DMEM for 3 h. The medium was subsequently exchanged with Neurobasal medium, and the cells were cultured for an additional 3 days. Stealth RNAi Negative Control Duplexes (Invitrogen) were used as a control.

Cell death assay

Neuronal cells were transfected with SelW siRNA or Stealth RNAi Negative Control Duplexes. The cells were incubated for 3 d, treated with H_2O_2 for 24 h, then analyzed with the *In Situ* Cell Death Detection Kit (Roche Applied Science, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was conducted as described in the manufacturer's instruction. In brief, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, incubated with TUNEL reaction mixture for 1 h at 37°C in the dark, and stained with DAPI. The TUNEL-positive and DAPI-stained cells were then visualized with fluorescence microscope. The percentage of apoptotic cells was calculated by dividing the TUNEL-positive neuronal cells by the total number of DAPI-stained neuronal cells.

RESULTS AND DISCUSSION

Developmental stage-associated mRNA and protein expression patterns of selenoproteins and non-selenium-containing antioxidants in the mouse brain

It has been reported that *selW* mRNA was expressed abundantly in the nervous systems of rat embryos at embryonic day 16 (E16) and E20 and in the hippocampus, cerebral cortex, dentate gyrus, and cerebellum of postnatal rat brains (Jeong et al., 2004). The age-dependent expression of brain antioxidants including selenoproteins were monitored using real-time PCR and Western blotting. The level of *selW* mRNA was compared with the levels of mRNAs which encode other selenoproteins and non-selenium-containing antioxidant enzymes. mRNA levels decreased in Trx, TR1, GPx, CAT, and SOD1 (58.4 ± 0.2%, 71.8 ± 0.1%, 56.5 ± 0.1%, 51.7 ± 0.1%, and 61.8 ± 0.1%, respectively) and remained constant in SOD2 in the mice of postnatal day 8 (P8) compared to E15. Only *selW* mRNA showed an increase (31.5 ± 0.2%) (Fig. 1). As shown in Fig. 2, protein levels of Trx, TR1, GPx1, and CAT increased slightly at P8, then decreased dramatically at P20. Almost all antioxidant enzymes decreased in the brain twenty days after birth. In contrast, SelW and SOD1 and SOD2 proteins significantly increased in P8 and P20 relative to E15, suggesting that these antioxidant proteins play an important role in the developing brain. These results are consistent with the previous studies which showed that both brain (Gu et al., 2000; Schweizer et al., 2004) and the central nervous system (Jeong et al., 2004) contain SelW.

Effects of SelW siRNA on H_2O_2-induced primary cerebral cortex neuronal cell death

Either SelW siRNA or Stealth RNAi Negative Control Duplexes were transfected into the primary cerebral cortex neuronal cells. Three days after transfection, the level of SelW expression was analyzed by RT-PCR and Western blot. Both *selW* mRNA and protein were effectively attenuated by SelW siRNA (Figs. 3A and 3B).

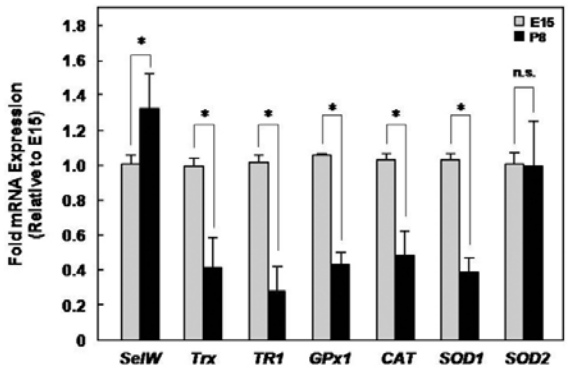

Fig. 1. mRNA level of selenoproteins and non-selenium-containing antioxidants in E15 and P8 mouse brain. Eight brains from embryos and three from postnatal mice were analyzed with real-time PCR. Five micrograms of total RNA isolated from the E15 and P8 mice brain were reverse transcribed and the resultant cDNA was amplified by using the TaqMan universal PCR master mix. GAPDH mRNA was used as reference in all samples. Data are represented as mean ± SD. Statistical significance was analyzed using Student's *t*-test. $^*P < 0.01$. n.s. not significant.

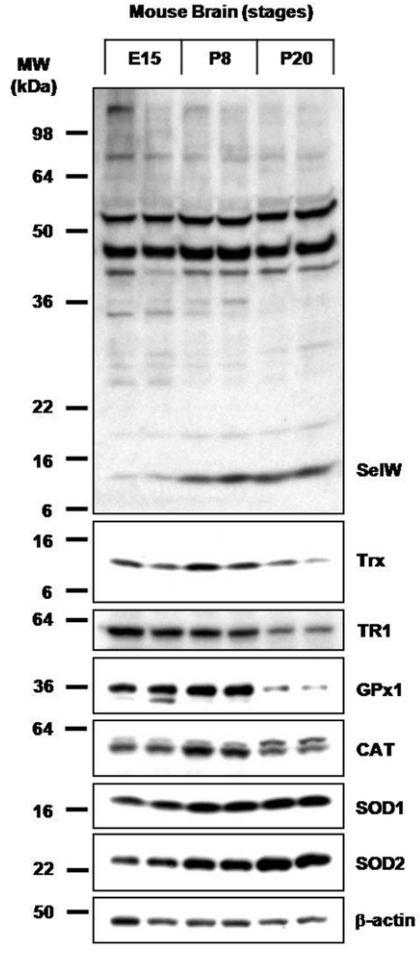

Fig. 2. Levels of selenoproteins and non-selenium-containing antioxidants in E15, P8, and P20 mouse brains. Whole brain lysates were subjected to immunoblot analysis with the indicated antibodies. β-actin was used to normalize expression level.

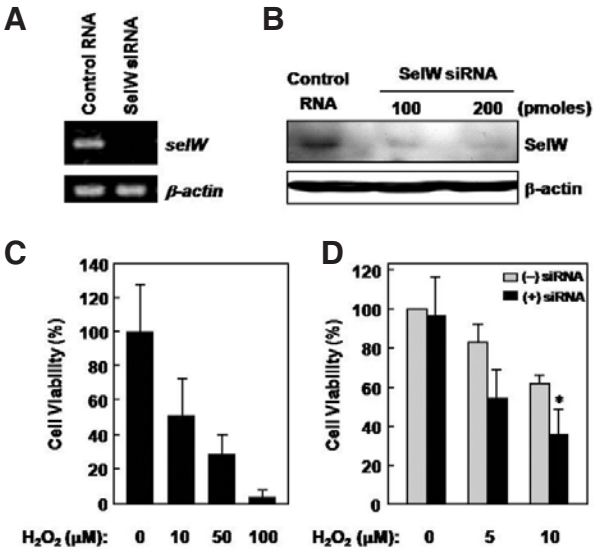

Fig. 3. Increased sensitivity of the SelW-suppressed cells to oxidant-induced cell death. The primary neuronal cells were transfected either with Stealth RNAi Negative Control Duplexes (Control RNA) or SelW siRNA. The *selW* mRNA and SelW protein were examined by RT-PCR and immunoblotting, respectively. (A) RT-PCR analysis was performed with primers specific for *selW* cDNA and *β-actin* cDNA as described previously (Jeong et al., 2002). (B) The level of SelW was determined by immunoblot analysis with anti-SelW antibodies. The blot was re-probed with anti-β-actin antibodies to verify protein loading. (C) Cerebral cortex-driven neuronal cells were treated with 10, 50 or 100 μM of H_2O_2 for 24 h and stained with DAPI. The cells showing normal shape of nucleus were then counted. (D) The cells were transfected with either SelW siRNA (200 pmoles) or with RNAi Negative Control Duplexes. Three days after transfection, the cells were treated with 5 or 10 μM of H_2O_2 for 24 h and the DAPI-stained cells were then analyzed for cell viability. Results are from at least three independent experiments. Data are represented as mean ± SD. Statistical significance between experimental and control RNA-transfected cells were analyzed using Student's *t*-test. $^*P < 0.01$.

The cascade reaction of neurotransmission results in the production of oxygen-derived free radicals, which can damage neurons (Pellmar, 1987). Both enzymatic and non-enzymatic antioxidant systems are essential for the protection of neuronal cells from these free radicals (Cohen, 1994; Rice, 2000). We previously reported that SelW functions as an antioxidant (Jeong et al., 2002). Recently, it was reported that SelW protects developing myoblasts from oxidative stress (Loflin et al., 2006). Here, we investigated the role of SelW in the cellular defense against oxidative damage in neurons. To test the vulnerability of primary cultured neuronal cells obtained from cerebral cortex to an exogenous oxidant, cells were treated with H_2O_2. Figure 3C shows that the viability of neuronal cells decreases as a function of increasing H_2O_2 concentration. When cells were incubated for 24 h in the presence of 10 μM H_2O_2, approximately 50% of the treated cells survived. However, when the concentration of H_2O_2 was elevated to 100 μM, less than 4% of the cells survived. Furthermore, the SelW siRNA transfected cells were more sensitive to exogenous oxidant than those obtained with Stealth RNAi Negative Control Duplexes (Fig. 3D). In the presence of 10 μM H_2O_2, 36% of siRNA-transfected neuronal cells were viable compared with

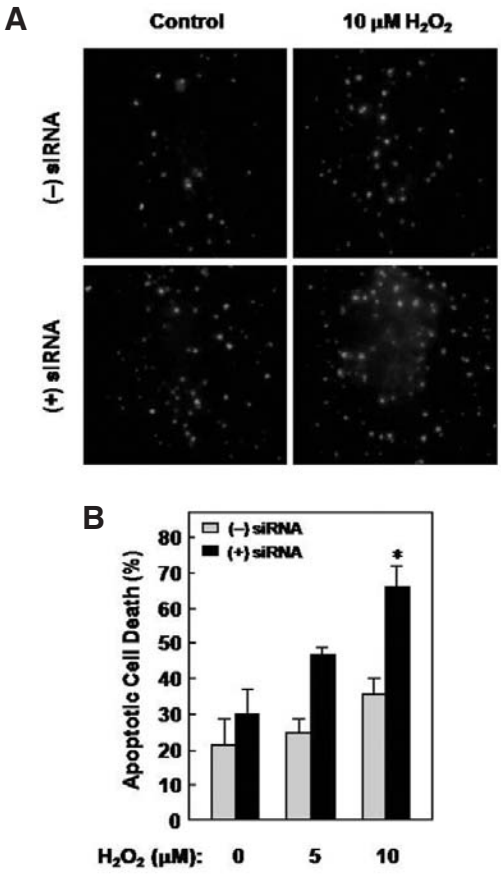

Fig. 4. Effect of SelW suppression on apoptotic cell death. (A) Treated neuronal cells were incubated with a TUNEL reaction mixture, followed by staining with DAPI. (B) The TUNEL-stained cells were analyzed as apoptotic cell death using fluorescence microscopy. The percentage of apoptotic cell death was calculated by dividing the number of TUNEL-positive cells by the total number of DAPI-stained cells. Results are from at least three independent experiments. Data are represented as mean ± SD. Statistical significance between experimental and control RNA-transfected cells were analyzed using Student's *t*-test. $^*P < 0.01$.

62% of control cells.

Using TUNEL staining method, we further monitored the extent of the H_2O_2-induced apoptosis and the effect of SelW down regulation on these neuronal cells. As shown in Fig. 4, the population of TUNEL-positive cells was about 10% higher in SelW siRNA-transfected cells compared with the control cells, even before the addition of H_2O_2. When these cells were treated with H_2O_2, the number of TUNEL-positive cells increased in a H_2O_2 dose-dependent manner. The extent of exogenous oxidant-induced cell death was approximately 2-fold higher in SelW siRNA-transfected cells compared within the control cells, suggesting that the attenuation of SelW expression due to siRNA renders the neuronal cells more sensitive to H_2O_2.

Like a number of newly identified selenoproteins, the physiological function of SelW is not well understood. The physiological function of SelW as an antioxidant has been suggested. When SelW was overexpressed in C6 rat glial cells, chinese hamster ovary (CHO) cells, and H1299 human lung cancer cells, sensitivity of these cells to exogenous oxidants was markedly decreased, suggesting that at high concentrations, SelW protects cells from oxidative stress-induced cell damage. Cells with SelW mutant in which a Sec residue (13^{th}) or a Cys residue (37^{th}) was substituted with serine, showed a decrease in their resistance to exogenous oxidants compared with wild-type cells. These results are consistent with the hypothesis that SelW functions as an antioxidant and Sec^{13} and Cys^{37} are crucial for this activity (Jeong et al., 2002). Our observation that the siRNA-induced knocked down of SelW in neuronal cells resulted in an increase in sensitivity to H_2O_2-induced oxidative stress provides definitive evidence supporting the antioxidant function of SelW. In addition, SelW was constitutively expressed in the brain at the embryonic and postnatal stages, and detected in the neuronal cells derived from the cerebral cortex. Together, our data suggest the possibility that SelW may play a crucial neuroprotective role in oxidative stress-induced primary neuronal cell damage, particularly during neuronal development.

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